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The effect of alternating influent carbon source composition on activated sludge bioflocculation

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Abstract

The impact of alternating influent carbon sources, i.e., glucose and starch, on activated sludge bioflocculation was investigated. To this end, four lab-scale reactors were operated during a long-term experiment. During this period the influent carbon source ratio (glucose/starch) was alternated every 7 or 35 days (i.e., a fast and slow switching frequency). Bioflocculation was monitored throughout the entire experiment using an extensive set of parameters, including macroscopic and microscopic activated sludge characteristics. Sludge hydrophobicity remained high (> 80%) throughout the experiment indicating good bioflocculation. However, sludge settleability decreased for all four reactors after a 60 day adaptation period to the applied alternation in influent carbon source. During this adaptation period, floc size decreased due to the release of microcolonies. The subsequent period was characterized by a decrease in settleability, coinciding with a release of primary particles and an increase in floc size. The observed phenomena could be linked with the protein concentration near the floc surface. This fraction mainly consists of hydrolytic enzymes necessary for the degradation of starch and is responsible for a progressive deterioration of the EPS matrix. The results of this specific study indicate to be independent of the influent carbon source ratio or switching frequency.

Keywords: activated sludge, carbon source, bioflocculation, floc structure, extracellular polymeric substances, hydrophobicity, shear sensitivity

1. Introduction

Formation of activated sludge flocs or **bioflocculation** is of major importance in achieving efficient solid-liquid separation in settling tanks for Conventional Activated Sludge systems (CAS) (Forster, 1976). Recently, activated sludge bioflocculation has also been linked to membrane fouling in membrane bioreactors (MBR) (Van den Broeck et al., 2010). Activated sludge flocs are built up by several particles, i.e., primary particles (0.5-5 µm) and microcolonies (5-13 µm) (Jorand et al., 1995; Klausen et al., 2004; Mikkelsen, 2001). Bioflocculation, starting from these *building blocks*, can be explained by several mechanisms: *(i)* bridging by extracellular polymeric substances (EPS) and cations *(ii)* hydrophobic interactions and *(iii)* charge neutralisation by Derjaguin-Landau-Verwey-Overbeek (DLVO-) type of interactions (Busch et al., 1968; Pavoni et al., 1972; Sobeck and Higgins, 2002; Urbain et al., 1993; Wilén et al., 2003).

As a consequence, assessing activated sludge bioflocculation is troublesome. At the moment, there is no unique measurement available, although, most often, conventional (macroscopic) parameters such as sludge volume index (SVI, [mL/g]) and effluent suspended solids (ESS, [mg/L]) are used. These parameters measure the consequence, e.g., settleability or effluent quality, of *good* or *bad* bioflocculated sludge. On the other hand, sludge characteristics such as EPS, surface charge and hydrophobicity, which are assumed to be key factors in the bioflocculation process, are also monitored (Liao et al., 2001; Liu and Fang, 2003; Sponza, 2002; Tian et al., 2011). However, a lot of contradictory data exist on the effect of these sludge characteristics on the bioflocculation process (Liu and Fang, 2003; Sheng et al., 2010). Next to this, floc strength measurements were introduced to assess floc stability (or strength) against break-up caused by high shear (shear sensitivity, K_{ss}) (Mikkelsen and Keiding, 2002) or a change in the chemical environment (dissociation constant) (Zita, 1994, Liao et al., 2002). The major drawback of the aforementioned measurements is that they are unable to directly assess the activated sludge bioflocculation condition. Monitoring flocs and floc size or in the case of deflocculation, floc break-up into smaller flocs and the subsequent release of microcolonies and primary particles, offers a more direct view on the (dynamics of the) bioflocculation process (Jarvis et al., 2005, Van Dierdonck et al., 2012b). This can be achieved by microscopic image analysis which has already proven its merits, e.g., in the filamentous bulking context (Arelli et al., 2009; da Motta et al., 2001; Jenné et al., 2007). However, monitoring activated sludge flocs and more specific, activated sludge primary particles and microcolonies in the context of bioflocculation has not received much attention yet.

Bioflocculation can be influenced by a multitude of factors including process parameters such as sludge retention time (SRT, [days]) (Massé et al., 2006; Liao et al., 2006), food-to-microorganism ratio (F/M, [gCOD/gMLSS.day]) (Barahona and Eckenfelder, 1984; Barbusinski and Koscielniak, 1995) and feeding regime (continuous or intermittent) (Bossier and Verstraete, 1996; Verachtert et al., 1980). Beside process parameters, also mixing intensity (Biggs and Lant, 2000), dissolved oxygen concentration (Palmgren et al., 1998) and temperature (Wilén et al., 2000) can affect activated sludge bioflocculation. Concerning the impact of influent on bioflocculation it is well-known that the cation content, more specific the ratio of mono-to-polyvalent ions, e.g., Na^+, K^+ versus Fe^{3+}, Al^{3+}, Ca^{2+}, Mg^{2+} has a significant impact through the formation of salt bridges (Sobeck and Higgins, 2002). This is known as the *Divalent Cation Bridging* theory (DCB) which states that polyvalent cations form bridges between the negatively charged functional groups (e.g., COO^-, OH^-) of EPS attached to different flocs/bacteria and in this way aggregate and stabilize the matrix of bacteria and EPS. If these polyvalent cations are replaced by monovalent cations, the activated sludge bioflocculation condition deteriorates. Novak et al. (1998) evaluated the cation balance from several industrial wastewaters and found that when the monovalent to polyvalent cations ratio (M/P-ratio) was smaller than two, better settling properties for the activated sludge were observed. On the other hand, not much research has been performed on the relation between influent carbon source and bioflocculation (Sponza, 2003, Ye et al., 2011).

In this research the effect of alternating influent carbon sources on bioflocculation is investigated. The rationale for this topic is that industrial wastewaters are often characterized by different product batches, e.g., one week product X is processed and the other week product Y or that there are seasonal variations. These changes can often be found in food and pharmaceutical industry or are induced by market driven production demands. Given that different carbon sources imply different break down mechanisms, the activated sludge requires some adaptation to the new carbon source. Such adaptations most probably have an impact on the activated sludge bioflocculation condition and, eventually, on sludge settleability. However, this topic has not been studied in much detail. Therefore, the effect of different *carbon source ratios* and *switching frequencies* on bioflocculation are investigated on lab-scale systems to gain more insight on the bioflocculation process under these influent conditions. Glucose and starch have been selected as carbon sources to invoke a clear difference in readily biodegradability since the latter requires enzymatic hydrolysis before being able to be consumed. As for the frequency in switching, we started from the rule of thumb that after 2 to 3 sludge residence times (SRT) the sludge is normally fully adapted to new influent or operational conditions. In practice, however, depending on the product batches, the switches might be more frequent. Therefore, we selected a switching frequency of 1/3 of the SRT to be contrasted with a switching frequency of 5/3 SRT. The former might be beneficial because the capacity to break down the previous carbon source might not have been lost completely yet. The latter might be beneficial because the sludge gets more time to adapt to the new carbon source. Finally, also the difference is studied between a complete switch to a new carbon source and a more limited switch, mimicking a complete production change or a partial switch of (some of) the processes. Starting with *well* bioflocculated sludge four lab-scale reactors were subjected to the aforementioned changes. While all classical macroscopic activated sludge characteristics were monitored, a major emphasis was put on the analysis of microscopic images of activated sludge since they reveal more of the fundamental dynamics underlying the bioflocculation process.

2. Materials and methods

2.1. Experimental set-up. Four 20 L lab-scale activated sludge systems (R1 - R4), each connected to a 5L conical settling tank, were seeded with activated sludge from a full-scale municipal wastewater treatment plant (Leuven, 130,000 population equivalents) which resulted in an initial mixed liquor suspended solids (MLSS) concentration of 3.71, 3.71, 3.83 and 3.53 g/L for R1 to R4, respectively. The reactors were continuously stirred with a magnetic stirrer and aeration was provided by means of porous aeration stones, yielding an oxygen concentration of over 4 mgO_2/L. The sludge retention time (SRT) was set to 21 days by controlling the daily amount of waste sludge. The reactors were initially operated with 100% glucose as carbon source in the influent for 155 days, which served as an extensive stabilization period (data not shown). On day 156 the contents of all reactors were mixed and equally distributed over the four reactors to ensure an identical activated sludge starting composition for all reactors. Starting from day 156 all activated sludge parameters (see Paragraphs 2.3, 2.5, 2.6, 2.7 and 2.8) were analyzed.

2.2. Influent. A synthetic influent was used to guarantee a stable and consistent feed to the reactor: 0.287 g/L K_2HPO_4; 0.133 g/L Na_2SO_4; 1.89 g/L KNO_3; 0.376 g/L $MgCl_2.6H_2O$; 0.888 g/L $CaCl_2$; 0.087 g/L $FeCl_3$ and 0.150 g/L yeast extract. With this influent the M/P-ratio was 1.5, thus, inducing *good* bioflocculation conditions for the sludge. The carbon source was, depending on the composition needed, 2.55 g/L glucose (= 0% starch), a mixture of 1.275 g/L glucose and 1.275 g/L starch (= 50% starch) or 2.55 g/L starch (= 100% starch), resulting in an influent COD of approximately 2800 mgO_2/L. The starch used as a carbon source was soluble starch derived from potatoes. An intermittent feeding pattern (5 L/d, administered in 8

aliquots of 625 mL per day) creating feast/famine cycles in the reactors was used to favor the growth of floc-forming bacteria, according to the kinetic selection theory (Verachtert et al., 1980, da Motta et al., 2002, 2003, Van den Broeck et al., 2009). This feeding pattern was applied to avoid interference of an excessive overgrowth of filamentous bacteria on settling and image analysis data. As a result, the obtained *good* or *bad* bioflocculation conditions can then solely be attributed to the switch in carbon sources. From day 156 to 183 all reactors were fed 100% glucose. From day 184 onward the influent changes were applied according to Figure 1.

A transition in carbon source ratio (%glucose / %starch) always starts from 100/0. A change from 100/0 to 0/100 or 50/50 is made. A further distinction is made by applying different periods between the transitions in carbon source ratio, i.e., every 7 days or every 35 days. This will be further referred to as switching frequency. As a result, the carbon source composition of reactor 1 (R1) is changed from 100/0 to 0/100 and for reactor 2 (R2) from 100/0 to 50/50, every 7 days. The carbon source for reactor 3 (R3) is changed from 100/0 to 0/100 and for reactor 4 (R4) from 100/0 to 50/50, every 35 days. In Figure 1 the applied changes are graphically illustrated, showing the percentage of starch in the influent versus the time of the experiment starting from day 156.

The applied switching frequencies of 1/3 and 5/3 SRT, corresponding to 7 and 35 days, were selected to obtain a different adaptation of the activated sludge to the change in influent carbon source composition.

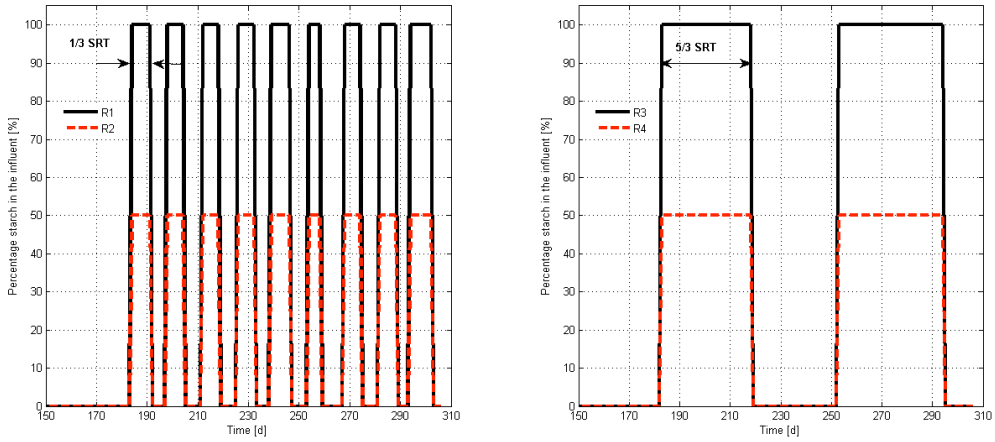

Figure 1 Schematic representation of the influent feeding regime for R1 and R2 (left) and for R3 and R4 (right).

2.3. General analytical methods. Chemical oxygen demand (COD) was measured with Hach Lange test cells (LCK 414, 614 and 014) on a spectrophotometer (DR 5000, Hach Lange). Dissolved oxygen in the 20 L bioreactors was measured using an LDO sensor (HQ40d, Hach Lange) and pH was measured with a PHC101 electrode (HQ40d, Hach Lange). Sludge volume index (SVI, [mL/g]), mixed liquor (volatile) suspended solids (ML(V)SS, [g/L]) and effluent suspended solids (ESS, [mg/L]) measurements were conducted in accordance to the procedures described in APHA Standard methods (Clesceri *et al.*, 1998).

2.4. Sludge samples. For image analysis and relative hydrophobicity, sludge samples were diluted with filtrated effluent to 1 g/L. Shear sensitivity was measured at 4 g/L. Undiluted sludge was used to perform EPS extractions, SVI and ML(V)SS measurements.

2.5. Image analysis. A fully automated image analysis procedure, ACTIAS (ACTivated sludge Image Analysis System), was used for the characterization of the activated sludge morphology (Jenné *et al.*, 2007). To this end, activated sludge images were captured manually using a light microscope (Olympus BX 51) with phase contrast illumination (Ph1) and a total magnification of 100 times. The microscope was equipped with a 3 CCD color video camera (Sony DXC-950P) which was connected to a computer. Microscopic images (80 images/day) were digitized and stored as JPG (768 x 576 pixels) using Zeiss KS 100.3 acquisition software. These images were subsequently processed by the developed image analysis procedure which is embedded in MATLAB Image Processing Toolbox 4.2 (The Mathworks Inc., Natick, MA). Through a consecutive segmentation and recognition algorithm, ACTIAS is able to calculate a specific set of morphological variables. An extensive description on how these variables are computed can be found in Jenné *et al.* (2007). In this research the focus was primarily on the object sizes. A distinction can be made between primary particles (0.5 to 5 µm) and microcolonies (5 to 13 µm) and flocs, being larger than 25 µm, after having assessed their total amounts, the percentage of primary particles (pp) and microcolonies (µcol) with respect to the total number of objects [%] is quantified.

2.6. Extracellular Polymeric Substances. EPS can be divided in two different classes, *(i)* soluble EPS or soluble microbial products (SMP) and *(ii)* bound or extractable EPS (eEPS). SMP are extracted and analysed according to Van Dierdonck et al. (2012a) and Van Dierdonck et al. (2012b), respectively.

2.7. Hydrophobicity. Hydrophobicity was quantified by means of the Microbial Adhesion To Hydrocarbons (MATH) method, developed by Rosenberg (1984) and frequently applied for estimating activated sludge hydrophobicity (Wilén et al., 2003). To this end, a 20 mL of a 1 g/L activated sludge sample 20 mL of n-Hexane (99%) was added. This mixture was emulsified for 2 min at 5000 rpm with a disperser (T25 digital Ultra-Turrax®, IKA). After emulsification, the sample was transferred to a separatory funnel where the two phases are allowed to separate for 5 min Subsequently a 15 mL sample of the aqueous phase was drawn off. The Relative Hydrophobicity (RH, [%]) is expressed as the ratio of MLSS concentration in the aqueous phase after emulsification ($MLSS_f$) to the MLSS concentration in the aqueous phase before emulsification ($MLSS_i$) (see Equation 1). For determination of the $MLSS_i$ a control sample (no n-Hexane added) is analyzed. Samples were analyzed in five-fold.

$$RH = \left[1 - \frac{MLSS_f}{MLSS_i}\right] \times 100 \qquad [\%] \tag{1}$$

2.8. Shear sensitivity. The method developed by Mikkelsen and Keiding (2002) was used to estimate the shear sensitivity of the sludge flocs, which is a measure for their stability. The shear sensitivity (K_{ss}) is defined as the equilibrium concentration of dispersed mass ($m_{d,\infty}$, [mg/L]) which would be reached after an infinite amount of time for a given amount of shear (G, [s^{-1}]), divided by the total concentration of suspended solids (m_T, [mg/L]). All shear tests are performed according to the method as described in Mikkelsen and Keiding (2002).

2.9. Statistical analysis.
Statistical dependence between two variables is investigated by performing a non-parametric rank correlation. The Spearman rank correlation (r_S) was used for this purpose. It assesses how well the relation between two variables can be described using a monotonic (in- or decreasing) function. Values for r_S lie between $-1 \leq r_S \leq 1$, with -1 indicating a perfect inverse correlation or 1, indicating a perfect positive correlation of the two variables. When there is little correlation between both variables the Spearman rank correlation coefficient has a value

near zero. Correlations are considered statistically significant at a 95% confidence interval ($p < 0.05$) (Neter et al.,1996).
To test the equality of parameters between different influent conditions among the four reactors after start-up an Analysis of Variance (ANOVA) is performed. T-tests were performed to investigate whether a significant difference exists between two conditions. Differences were considered statistically significant at a 95 % confidence interval ($p < 0.05$) (Neter et al., 1996). Statistical analysis is carried out with the Matlab Statistics Toolbox 6.2. (The Mathworks Inc., Natick, MA).

3. Results and Discussion

3.1. 100% glucose starting position in all four reactors

On day 156 the contents of all reactors were mixed and equally distributed over the four reactors. Prior to applying the changes in influent conditions, the reactors were fed with 100% glucose as carbon source from day 156 to day 183. Also, starting from day 156 the sludge was extensively monitored. Based on the data from this period, a comparison between all reactors was made by means of an analysis of variance. This was done to investigate if the reactors could be considered similar in terms of activated sludge composition, before applying the changes in influent conditions. This period is indicated by G1 on all subsequent figures. The results of the analysis of variance revealed that for soluble effluent COD; image analysis parameters; shear sensitivity; hydrophobicity; extracellular polymeric substances no significant differences were found. The values found for SVI were significantly different at 5 %. However, they do not differ much from each other and are in the same range (R1 = 52.7 ± 4.0; R2 = 46.6 ± 2.4; R3 = 50.1 ± 3.4 and R4 = 44.6 ± 2.9 mL/g). Also, ESS and effluent COD values were higher for R3 and R4 than the values obtained for R1 and R2. Therefore, ESS and effluent COD will not be used for quantitative data analysis, but will be mentioned in a qualitative sense.
Starting from day 184 the aforementioned changes in influent conditions were applied. The data collected throughout the experiment will be analysed and discussed as a whole per reactor. No further comparison within one reactor will be made between periods fed with glucose, starch or glucose-starch as carbon source because the main goal of the experiment is to gain information on the resulting overall bioflocculation condition of the sludge due to the specifically applied influent conditions.

3.1. Observed effects

Starting from conventional macroscopic parameters, a decrease in sludge settleability is observed for all four reactors, as measured by an increase in SVI. The increase in SVI is presented in Figure 2.

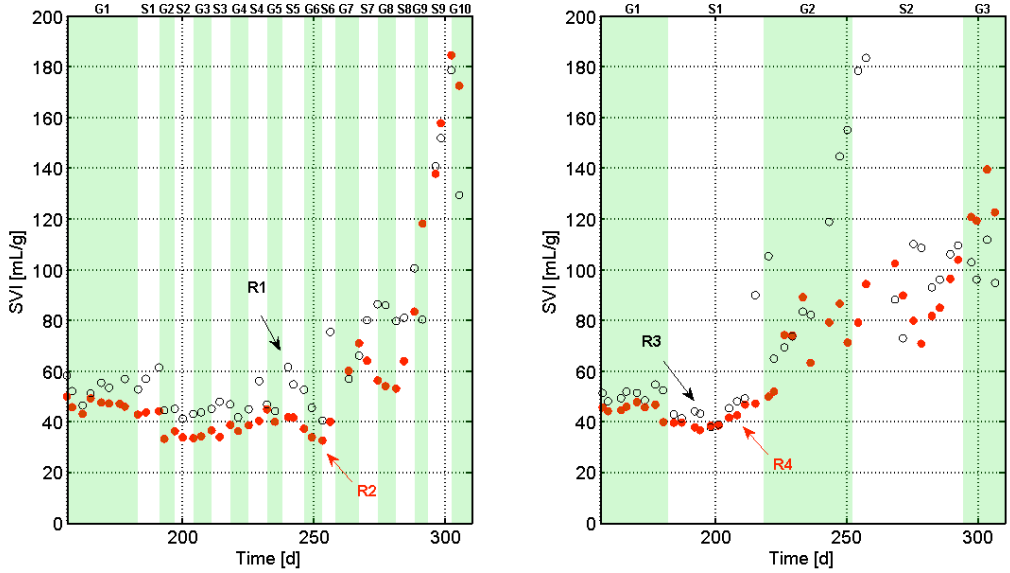

Figure 2 Evolution of the sludge volume index resulting from the carbon source conditions applied to the reactors. All reactors show an increase in SVI after an adaptation period to the influent conditions. (left) SVI for 1/3 SRT switching frequency for R1 between 100/0 to 0/100 (black) and for R2 from 100/0 to 50/50 (red). (right) SVI for 5/3 SRT switching frequency for R3 between 100/0 and 0/100 (black) and for R4 between 100/0 to 50/50 (red).

The graph on the left shows the evolution of SVI for R1 and R2, the one on the right for R3 and R4. Two x-axis are added to the graph(s). The bottom x-axis shows the time duration of the experiment in days (150 to 310), while the top x-axis makes the corresponding distinction between the applied carbon source ratios. G1 to G10 (for R1 and R2) or G1 to G3 (for R3 and R4) correspond to the periods when the influent carbon source ratio is 100/0 glucose/starch. S1 to S9 (for R1 and R2) or S1 to S2 (for R3 and R4) corresponds to the periods when the influent carbon source ratio is 0/100 or 50/50. From the left graph on Figure 2 it can be inferred that when alternately changing the influent composition every 7 days an increase in SVI is observed starting from G6 (about 60 days after starting the alternating feeding pattern). From G1 to G6 values remain constant and low at 50.17 ± 6.07 and 41.16 ± 5.20 mL/g for R1 and R2, respectively. For reactors R3 and R4 (right graph on Figure 2), the increase in SVI started from G2 onwards (more than 60 days after starting the alternating feeding pattern). During period G1 and S1 SVI values remain constant and low at 49.45 ± 11.17 and 42.85 ± 3.72 mL/g for R3 and R4, respectively. Based on the measured SVI values it can be deduced (T-test) that the SVI for R1 and R3 (0/100) is significantly higher than for R2 and R4 (50/50). Experiments performed by Martins et al. (2011) also show that sludge fed with starch exhibits higher SVIs than when fed with glucose or, in this case a 50 % glucose - 50 % starch mixture. In addition, the final SVIs for R1 (129.3 mL/g) and R2 (172.3 mL/g) are higher than for R3 (94.7 mL/g) and R4 (122.5 mL/g), indicating that, although the ultimate effect is the same, changing the carbon source every 5/3 SRT (or every 35 days) seems to have a less drastic effect on settleability. The 60-day period (3x SRT), preceding the changes in SVI, can be labelled as an adaptation period during which the activated sludge is getting used to the imposed influent conditions and involves changes in production of enzymes, e.g., α-glucosidase, and changes in bacterial community to be able to cope with the degradation of starch. Starting from period G6 for R1 and R2 and G2 for R3 and R4 activated sludge settleability is impaired, as indicated by the significant increase in SVI.

Although settleability worsens in all four reactors, this only results in a small increase in the ESS (Figure 3) for R2 and R4. For R1 and R3 even a downward trend can be observed.

Similar to SVI, this trend is also linked to the carbon source composition, but not to the switching frequency. A similar observation can be made for the effluent COD (CODeffl, [mgO2/L]) (data not shown).

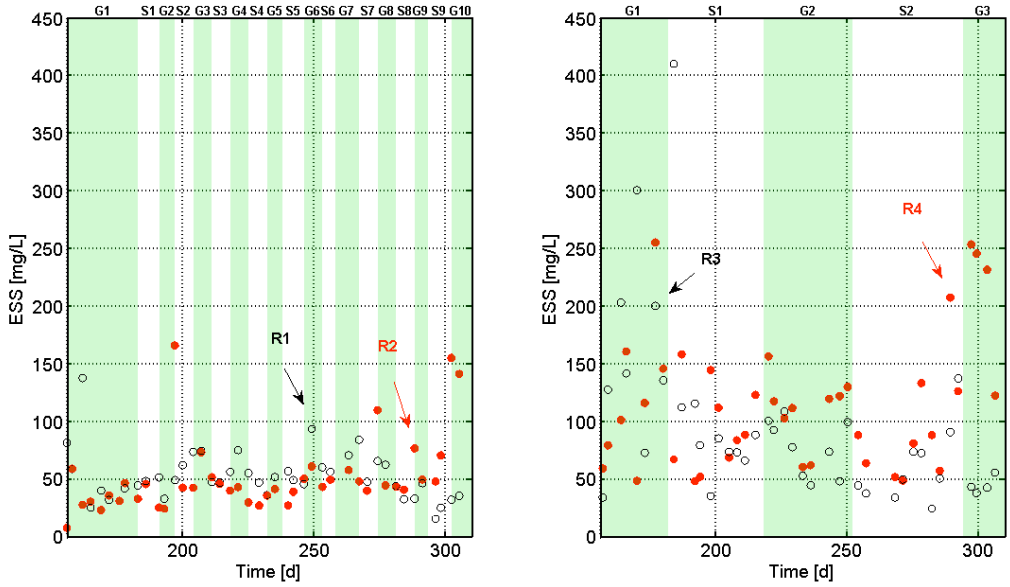

Figure 3 Evolution of the effluent suspended solids resulting from the carbon source conditions applied to the reactors. (left) ESS for 1/3 SRT switching frequency for R1 between 100/0 to 0/100 (black) and for R2 from 100/0 to 50/50 (red). (right) ESS for 5/3 SRT switching frequency for R3 between 100/0 and 0/100 (black) and for R4 between 100/0 to 50/50 (red).

In literature the SVI and the ESS are often linked to the activated sludge's bioflocculation condition. According to this reasoning, efficient settling which is characterized by low SVI and ESS concentration, is caused by large and dense flocs or, thus, well- bioflocculated sludge. The reverse holds for high SVI and ESS concentration (Liao et al., 2001). In this case, however, settleability deteriorates in all reactors, although ESS concentration does not increase for all four reactors. This indicates that SVI and ESS do not always result in an accurate estimation of the activated sludge's bioflocculation condition. In addition, this also suggests that bioflocculation and activated sludge settling are different processes which are not always clearly linked. The information obtained from parameters related to activated sludge settleability should, therefore, be handled with care when using them to assess the activated sludge's bioflocculation condition. The information derived from these parameters should then be complemented with other measurements, such as, image analysis (Van Dierdonck et al., 2012b).

3.2. Causes for the deteriorated settleability

Image analysis revealed that the increase in SVI was not caused by an excessive growth of filamentous bacteria. However, the amount of filaments did slightly increase in comparison to the start of the experiment. The amount of filamentous organisms and their protruding length was, by far, too low to cause bulking. The average filament length (in pixels) of the sludge flocs during the period prior to the changes in influent conditions (G1) and the average filament length for the remainder of the experiment are shown in Table 1. Suppression of excessive growth of filamentous bacteria was obtained by applying an intermittent feeding pattern.

Table 1 Average filament length (in pixels, ± standard deviation) for all four reactors during the period prior to the changes in influent conditions (A) and under changing influent conditions (B).

	R1	R2	R3	R4
A	322±194	440±320	341±209	389±184
B	741±686	655±478	784±539	782±510

On the other hand, it is possible that by alternately changing the influent composition, the structure of the activated sludge flocs changes. This could result in, and help explain, the deteriorated settling properties. The structure of activated sludge can be monitored in terms of floc size (equivalent diameter D_{eq}, [µm]), relative primary particle (pp) and relative microcolony (µcol) amount ([%]) (Figure 4). These activated sludge building blocks give more information on the bioflocculation condition of the sludge (Van den Broeck et al. 2010; Van den Broeck et al. 2011; Van Dierdonck et al., 2012b).
The evolution of the equivalent diameter, relative primary particle and microcolony amount throughout the experiment are presented in Figure 4.

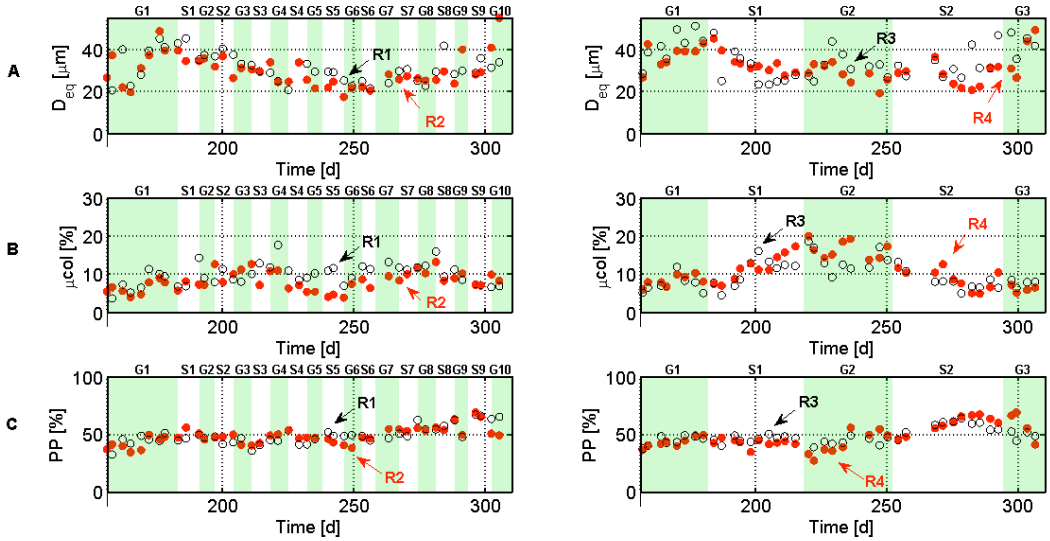

Figure 4 Evolution of the equivalent diameter (Deq, [µm]) (A), relative microcolony amount ([%]) (B) and relative primary particle amount ([%]) (C) resulting from the carbon source conditions applied to the reactors. (left) values for 1/3 SRT switching frequency for R1 between 100/0 to 0/100 (black) and for R2 from 100/0 to 50/50 (red). (right) values for 5/3 SRT switching frequency for R3 between 100/0 and 0/100 (black) and for R4 between 100/0 to 50/50 (red).

From Figure 4 (A and B), the initial decrease in D_{eq} (deflocculation) for all reactors can be explained by the release of microcolonies during the adaptation period. R1 and R2, however, do not show this trend, although their floc size also decreases. The observations are in line with the data obtained by Martins et al. (2011). He mentions that starch based systems are dominated by smaller flocs, and stimulate the formation of microflocs. The deflocculation of large flocs to smaller flocs could be the result of a more efficient substrate acquisition when the system is not fully adapted to the applied influent conditions (Wanner, 1994). Although the increase in microcolonies is not extremely high, it has a significant effect on the Deq of

the sludge flocs. However, this release of microcolonies had no significant effect on settleability, ESS and CODeffl. Most probably microcolonies are still large enough (5 - 13 µm) to allow for efficient settling. Consequently, they can be retained in the system as there is no increase in ESS or CODeffl observed. In addition, floc strength was not affected by the release of microcolonies. Similar observations can be made for R1 and R2. However, in this case, microcolony data is subject to more up and downward trends starting from G1 until G8, as a result of the frequent changes in carbon source.
Subsequent to this period, characterized by a decrease in the equivalent diameter, an increase in floc size and decrease in the relative amount of microcolonies till the original level can be observed for all reactors. During this period, starting from G6 and S2 onwards for R1, R2 and R3, R4, respectively, the relative amount of primary particles increases (Figure 4C). This period coincides with the deteriorating settleability of the sludge.
Correlations between the morphological characteristics of the floc (i.e., floc size, primary particles and microcolonies) and SVI are made. The goal was to investigate whether it is indeed a change in floc structure which induces the decreased settleability. In Table 2 the Spearman rank correlation coefficients (r_S) and corresponding p-values can be found. If the p-value is less than 0.05, the correlation between both parameters is significant.

Table 2 Spearman correlation (r_S) coefficients and p-value (between brackets) between SVI and D_{eq}, primary particles (pp) and microcolonies (µcol) for R1 to R4. Correlations are considered statistically significant at a 95% confidence interval ($p < 0.05$).

	SVI			
	R1	R2	R3	R4
D_{eq}	0.15 (0.382)	0.30 (0.0743)	-0.23 (0.889)	-0.30 (0.064)
pp	0.68 (<< 0.05)	0.45 (0.006)	0.33 (0.0451)	0.52 (0.009)
µcol	-0.048 (0.773)	0.074 (0.663)	0.14 (0.400)	-0.15 (0.370)

From Table 2 it can be inferred that the increase in SVI is significantly positively correlated with the relative amount of primary particles present in R1, R2, R3 and R4. This correlation suggests that there is a link between the observed SVI values and the relative amount of primary particles. It means that high SVI values are correlated with high amounts of primary particles. Since the correlation coefficients are not extremely high, it could be suggested that an increase in primary particle amount can only partly explain the increase in SVI. Most likely, other factors also influence the settleability of the sludge. On the other hand, no correlation with D_{eq} or the relative amount of microcolonies with SVI could be found. In addition, the correlation between the relative amount of primary particles released and floc stability, as measured by the shear sensitivity (K_{ss}) was assessed. For R1 and R4 significant positive correlations were found, but not for R2 and R3.

K_{ss}, however, correlates well with the initial dispersed mass ($m_{d,0}$) present in the sludge, which is composed of primary particles and/or microcolonies (R1: $r_S = 0.56$, $P = 0.0002$; R2: $r_S = 0.50$, $P = 0.0016$; R3: $r_S = 0.52$, $P = 0.0008$; R4: $r_S = 0.54$, $P = 0.0004$). This indicates that if a significant amount of dispersed particles is present in the sludge than the sludge has a low resistance to break-up by shear. From the above it is clear that based on only SVI and ESS data, it is not possible to assess the bioflocculation condition of the activated sludge. Additional information, which can be provided by image analysis, aids in clarifying the bioflocculation dynamics going on at the level of the floc.

3.3 Primary particle release

The cause for the release of primary particles from the sludge was investigated by monitoring the hydrophobicity ([%]) of the sludge and by analyzing the EPS composition (polysaccharides and proteins, [mg/gMLVSS]).

Hydrophobicity. In general, high sludge hydrophobicity is linked to a better bioflocculation condition of the sludge (Urbain et al., 1993; Jorand et al., 1995; Liao et al., 2001; Liu and Fang, 2003; Xie et al., 2010). When following the previous reasoning on settleability and bioflocculation, a high hydrophobicity should then be linked to a low SVI and ESS concentration. Thus, based on the obtained bioflocculation data, a decrease in hydrophobicity is expected when the activated sludge settleability deteriorates.

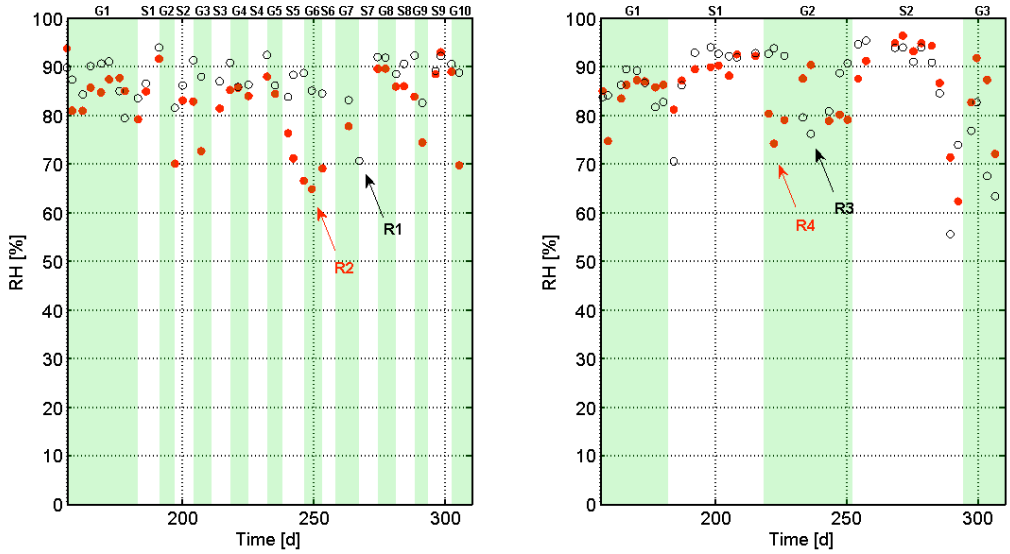

Figure 5 Evolution of the hydrophobicity ([%]) resulting from the carbon source conditions applied to the reactors. (left) Hydrophobicity for 1/3 SRT switching frequency for R1 between 100/0 to 0/100 (black) and for R2 from 100/0 to 50/50 (red). (right) Hydrophobicity for 5/3 SRT switching frequency for R3 between 100/0 and 0/100 (black) and for R4 between 100/0 to 50/50 (red).

The values of the activated sludge's hydrophobicity throughout the experiment (Figure 5) remain high for all reactors (87.2 ± 4.4 % forR1; 81.9±7.6 % forR2; 85.4±9.5 % for R3 and 85.2 ± 7.5 % for R4). Spearman rank correlation coefficients are not significant between hydrophobicity and SVI. Furthermore, no significant correlation between hydrophobicity and primary particle amount or floc strength could be obtained. In this case, sludge hydrophobicity does not seem to be linked to the release of primary particles and the subsequent increase in SVI or decrease in floc stability as measured by Kss.

EPS, and more specific its protein content, is an important factor governing activated sludge hydrophobicity (Urbain et al., 1993, Jorand et al., 1995). No significant correlation between protein content of the SMP fraction (SMP_{PN}) and sludge hydrophobicity could be found, except for R3 (r_S = 0.43, P = 0.0078). For R1 (r_S = 0.40, P = 0.0126), R3 (r_S = 0.36, P = 0.0245) and R4 (r_S = 0.41, P = 0.0106) a positive correlation between RH and $eEPS_{PN}$ was found. The correlation for R2 was not significant but the data indicates the same positive trend (r_S = 0.25, P = 0.143). For both polysaccharide fractions (SMP_{PS} and $eEPS_{PS}$) no significant correlations with sludge hydrophobicity were found. These results indicate that the $eEPS_{PN}$ fraction delivers an important contribution to RH, notwithstanding that other factors

such as microbial community (Jorand et al., 1995) and applied process conditions (Liu and Fang, 2003) also play a role in determining activated sludge hydrophobicity.

EPS. In Table 3 the concentrations of the different EPS fractions and their constituents are summarized. A distinction between the period G1 (before the red line), when all reactors were fed a carbon source ratio of 100/0, and the average over all applied influent changes, is made. From the data it can be inferred, that despite the differences in applied influent conditions, the composition of EPS is very similar for all reactors and the amount of SMP_{PN} for all reactors remained constant.

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Table 3 Average EPS concentrations (± standard deviation) expressed in mg/g MLVSS for all four reactors from the period *(i)* before applying the changes in influent conditions (G1 = 100% glucose as carbon source) and (ii) when the activated sludge was subject to the changes in influent conditions, corresponding to an influent carbon source ratio of 0/100 or 50/50 (glucose/starch) and different switching frequencies (1/3 SRT for R1 and R2; 5/3 SRT for R3 and R4).

	R1		R2		R3		R4	
	[mg/gMLVSS]							
	G1	0/100	G1	50/50	G1	0/100	G1	50/50
SMP_{PN}	1.7 ± 0.5	2.0 ± 0.5	1.6 ± 0.2	1.9 ± 0.5	1.6 ± 0.4	2.0 ± 0.6	1.5 ± 0.5	1.9 ± 0.4
SMP_{PS}	10.4 ± 3.9	10.0 ± 3.9	9.8 ± 1.9	10.6 ± 4.9	9.7 ± 5.6	10.1 ± 4.5	7.7 ± 3.1	8.5 ± 2.5
SMP_{total}	12.1 ± 4.2	11.9 ± 4.3	11.5 ± 2.0	12.5 ± 5.1	11.3 ± 5.6	12.1 ± 4.6	9.2 ± 3.2	10.4 ± 2.4
$SMP_{PN/PS}$	0.18 ± 0.06	0.22 ± 0.08	0.17 ± 0.03	0.21 ± 0.09	0.20 ± 0.06	0.19 ± 0.09	0.20 ± 0.07	0.22 ± 0.09
$eEPS_{PN}$	22.1 ± 2.0	30.0 ± 3.5	22.5 ± 2.5	27.9 ± 3.5	20.4 ± 4.5	31.1 ± 5.9	20.6 ± 4.3	28.2 ± 3.3
$eEPS_{PS}$	55.1 ± 6.2	49.3 ± 7.1	52.9 ± 4.6	54.7 ± 10.7	55.7 ± 7.8	58.0 ± 9.9	50.9 ± 21.0	58.0 ± 6.9
$eEPS_{total}$	77.2 ± 7.9	79.3 ± 7.5	75.4 ± 6.5	82.6 ± 11.8	76.1 ± 10.7	89.15 ± 12.5	71.6 ± 18.6	86.2 ± 7.5
$eEPS_{PN/PS}$	0.40 ± 0.03	0.62 ± 0.11	0.43 ± 0.04	0.54 ± 0.14	0.40 ± 0.06	0.56 ± 0.14	0.32 ± 0.2	0.49 ± 0.08
EPS_{total}	89.3 ± 6.2	91.3 ± 8.6	86.8 ± 6.3	95.1 ± 13.1	87.4 ± 8.9	101.2 ± 14.7	80.8 ± 18.6	96.6 ± 8.1

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Soluble Microbial Polymer (SMP) constituents (SMP_{PN}, SMP_{PS}, $SMP_{PN/PS}$-ratio and total amount) do not show significant correlation with SVI or the amount of primary particles. The protein concentration of the extracted EPS ($eEPS_{PN}$), however, is positively correlated with SVI, for all four reactors. This indicates that, besides the amount of primary particles, also this protein fraction plays an important role in determining the sludge settleability (Urbain et al., 1993). The Spearman rank correlation coefficients are r_S = 0.50 (P = 0.0015), r_S = 0.38 (P = 0.0212), r_S = 0.40 (P = 0.0140) and r_S = 0.61 (P = 0.0001) for R1, R2, R3 and R4, respectively. The correlation is visualized in Figure 6 (right). In addition, for all four reactors a significant increase (approximately 50%) in eEPS protein concentration can be observed throughout the experiment. This increase ranges from 22.1 ± 2.0 to 32.9; 22.5 ± 2.5 to 29.7; 20.4 ± 4.5 to 32.9; 20.6 ± 4.3 to 28.2 mg/gMLVSS, for R1, R2, R3 and R4, respectively (Figure 6). These are the average values of the concentration before and after the start of the changing influent conditions, as indicated by the red line.

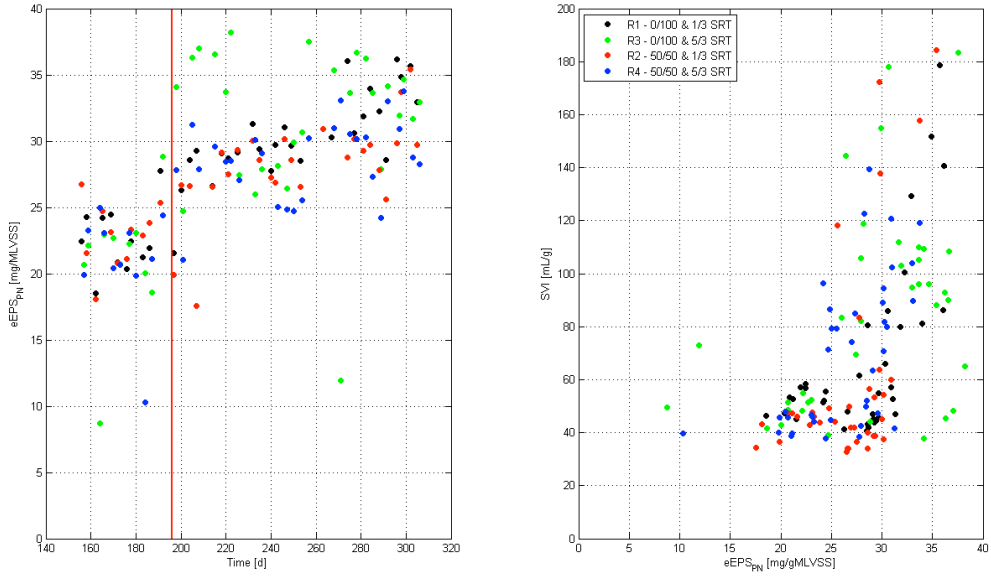

Figure 6 (left) Evolution of $eEPS_{PN}$ throughout the experiment for all four reactors. The vertical red line indicates the starting point for the changes in influent conditions. (right) positive correlation of $eEPS_{PN}$ versus SVI, indicating an increase in SVI with increasing $eEPS_{PN}$ concentration when alternately changing the influent carbon source between glucose and starch for various ratios (0/100, 50/50 and 0/100) and time periods (expressed in SRT).

In addition, $eEPS_{PN/PS}$ is often used as an indicator for bioflocculation (Liao et al., 2001; Van Dierdonck et al., 2012b). Although values for $eEPS_{PN/PS}$ are not extremely high, for all reactors an increase could be observed when the changes in influent conditions were applied (see Table 3). The increase in $eEPS_{PN/PS}$ ratio is mainly due to the increase in $eEPS_{PN}$ concentration. However, given the release of the primary particles in the final phase, in this experiment an elevated $eEPS_{PN}$ and corresponding elevated $eEPS_{PN/PS}$ does not indicate an improvement in the activated sludge's bioflocculation condition. This increase suggests that, the microbial community is adapting to the changes in carbon source. Production of (other) enzymes, which are proteins necessary to degrade both types of carbon source are being produced. The latter has already been observed by (Nybroe et al., 1992; Ye et al., 2011; Martins et al., 2011). Studies have shown that the dominant mechanism for starch removal is adsorption with subsequent enzymatic hydrolysis inside the flocs (Karahan et al., 2006, Martins et al., 2011). In this case, the $eEPS_{PN}$ concentration is significantly correlated to the amount of primary particles present in the mixed liquor. For R1 (r_S = 0.63, P = << 0.05), R2

(r_S = 0.48, P = 0.0029) and R4 (r_S = 0.37, P = 0.0223) a significant positive correlation is found. However, for R3 no significant correlation could be found, but the same positive trend can be observed in the data (r_S = 0.31, P = 0.0603).
The observed release in primary particles, coinciding with a deteriorating settleability, can then be explained by the progressive degradation of the EPS matrix. This is done through the action of the accumulated hydrolytic enzymes near the floc surface ($eEPS_{PN}$). Hydrolysation of the EPS matrix surrounding the floc can then result in the release of primary particles. Such a phenomenon has already been observed under different conditions by Wilén et al. (2000); Akhurst et al. (2002) and Ayol et al. (2008). However, besides the release of primary particles, floc size also increased, which is contradictory to the release of the primary particles.
Two explanations for this increase in floc size can be put forward. The first one involves swelling of the EPS network. Since this EPS network is highly negatively charged and these negative charges are surrounded by counterions, the resulting osmotic gradient may lead to high water uptake and thus swelling of the EPS matrix (Jin et al., 2004). Another explanation can be found in the reincorporation of microcolonies in the activated sludge flocs. The data presented on Figure 4A and 4B suggest that increase in floc size coincides with a decrease in microcolonies. It is possible that through the action of shear these particles are brought into close contact with each other and can then reflocculate. Based on the data, it is not possible to exactly determine why the floc size starts to increase again. On the other hand, Martins et al. (2011) reported that sludge flocs grown on starch appear to be more porous, less compact and smaller in size. This could also be qualitatively assessed (except for the smaller size) based on the activated sludge images taken throughout the experiment.

4. Conclusions

This study investigated the impact of alternating in influent carbon source, i.e., glucose and starch, on the bioflocculation condition of activated sludge. To this end, four reactors were operated under different conditions. Two reactors received every 7 days (1/3 SRT) a different influent carbon source ratio going from 100/0 (glucose/starch) to 0/100 (glucose/starch) (R1) or 50/50 starch (R2). The same variation was applied for R3 and R4, respectively, but the switching frequency between to conditions was set to 35 days (5/3 SRT).
The obtained results concerning sludge bioflocculation are independent of the applied influent conditions (ratio and switching frequency). All reactors experienced an adaptation period when the alternating influent conditions were applied which lasted for approximately 60 days (or approximately 3 SRTs). During this period floc size decreased as a result of the release of microcolonies (deflocculation). SVI remained low and constant during this period. The subsequent period was characterized by a decreasing settling capacity (increase in SVI), a significant release of primary particles and an increase in floc size and $eEPS_{PN}$. Also higher values for shear sensitivity were measured. These indicated that the applied influent conditions negatively affected the resistance of the flocs against shear. Furthermore, a significant increase in the $eEPS_{PN}$ fraction by approximately 50% was observed. This could be mainly attributed to the accumulation of hydrolytic enzymes near the floc surface. These hydrolytic enzymes resulted in a progressive degradation of the eEPS matrix which was evidenced by the release of primary particles. At the same time, floc size was increasing, which can be the result of a swelling of the EPS or a re-incorporation of microcolonies in the floc. Sludge hydrophobicity, however, remained high throughout the entire experiment and could be mainly attributed to the $eEPS_{PN}$ fraction. The deteriorating settling capacity, in this case, could be related to the amount of primary particles and $eEPS_{PN}$ concentration. Interestingly, all reactors responded in the same manner despite the different influent conditions applied, indicating that the time between different influent carbon sources and their ratios did not play a significant role in the overall performance of the system that we studied here. Notice, however, that the results might differ if the difference in carbon source is not *merely* related to the fact that one of them requires hydrolytic activity before being able to be consumed but would require other internal metabolic pathways to be activated. Furthermore, if one would wait 2 to 3 sludge residence times, chances are higher to reach

(each time) again a more beneficial steady-state. The transients in between might, however, be very long such that the average performance over a long period would still be moderate.

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